Distinguishing between Two Closely Related Strains of *Xylella fastidiosa*

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Abstract: A real time PCR assay (rt-PCR), using Taqman minor groove binder (MGB) probes was developed to quantify and differentiate two strains of *Xylella fastidiosa*, Pierce's Disease (PD) strain and Almond Leaf Scorch (ALS) strain. Two isolates, Temecula and Dixon, were used as references for the PD strain and the ALS strain, respectively. Primers and probes based on the gyraseB sequence were able to distinguish between both the isolates, and DNA concentrations as low as 1.01×10^{-5} ng/µl (3 copy number/µl) were detected. MGB probes were also able to distinguish between the Temecula and Dixon isolate DNA from a mixture of the two DNA's. Whereas the Temecula specific MGB probe showed 10^6–fold resolution, the Dixon specific probe showed only 10-fold resolution. We have developed a reliable, sensitive and specific rt-PCR assay using MGB probes to quantify and distinguish DNA of the two strains of *X. fastidiosa*.

Keywords: Real time-PCR, *Xylella fastidiosa*, strains, PD, ALS, Temecula, Dixon.

I. Introduction

Xylella fastidiosa [1] is a gram negative, xylem-limited, fastidious and non-flagellated bacterium with a number of strains that infect both monocotyledon and dicotyledon plants from 28 families. Specific strains are each associated with certain important crop diseases including Pierce's Disease (PD) in grapevines, citrus variegated chlorosis (CVC) in citrus, phony peach disease (PPD), as well as periwinkle wilt and leaf scorch in plum, elm, maple, oak, sycamore and coffee [2]. This pathogen is transmitted by leafhoppers commonly known as "sharpshooters" and "spittle bugs." Among the insect vectors, the principle vector of *X. fastidiosa* is the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* [3], [4].

Different strains of *X. fastidiosa* vary in their host-plant range and pathogenesis. All of these strains have been grouped in five different clades: PD, Oleander Leaf Scorch (OLS), Almond Leaf Scorch (ALS), Mulberry (hardwood) and CVC. These clades were derived from morphological and molecular attributes of the bacteria such as biofilm formation and morphology, repetitive extragenic palindromic PCR, randomly amplified polymorphic DNA-PCR, CHEF analysis, enterobacterial repetitive intergenic sequence-PCR, and restriction fragment length polymorphism [5]-[9]. With the use of DNA-DNA relatedness assays and the 16S-23S intergenic spacer (ITS) sequences of 26 strains, Schaad et al. [10] divided *X. fastidiosa* into three distinct groups: (a) *X. fastidiosa* subsp. *fastidiosa* (b) *X. fastidiosa* subsp. *multiplex* (c) *X. fastidioa* subsp. *pauca*. Later, Schuenzel et al. [11] suggested another subspieces, *X. fastidiosa* subsp. *sandyi,* based on multigene phylogenetic studies. More recently the gyraseB gene has been used to distinguish between *X. fastidiosa* strains [9], [12]. GyraseB is a faster evolving gene than the 16S gene which makes it a better candidate to discriminate between different bacterial strains.

Methods such as ELISA [13], PCR [7], [14] and real time (rt)-PCR [15] have been used for the detection of *X. fastidiosa* infection. Of these, rt-PCR based on 16S and ITS regions is the most sensitive and was employed for early detection of *X. fastidiosa* infection [15]. *X. fastidiosa* displays strain specificity in its range of infection and pathogenesis. The PD strain, when inoculated into almond plants elicited pathology consistent with ALS. However, when the ALS strain was inoculated into grape plants, bacteria did not multiply sufficiently to cause Pierce's Disease [16]. These strains also showed variation in their association with the GWSS. Almeida and Purcell [17] reported that the rate of PD strain acquisition by the GWSS from infected grape plants was three times that of the ALS strain acquisition from infected almond plants.

Clearly, strain variation amongst *X. fastidiosa* plays a critical role in pathogenesis; therefore an efficient method for detection of specific strains would be helpful in determining *X. fastidiosa* epidemiology. The method developed by Schaad et al. [15] can detect 1 copy number/µl, however, it does not differentiate between different *X. fastidiosa* strains. Minor Groove Binder (MGB) probes can be used in rt-PCR to increase the specificity needed to distinguish between strains. MGB probes are dual labeled Taqman probes, with a minor groove binder at the 3'end. This minor grove binder increases the T_m of the probe, hence the specificity is increased [18], [19].

In this study a rt-PCR method using MGB probes was developed to distinguish between two closely related strains of *X. fastidiosa*- PD strain and ALS strain. The Temecula and Dixon isolates were used as a reference for the PD and ALS strains, respectively.

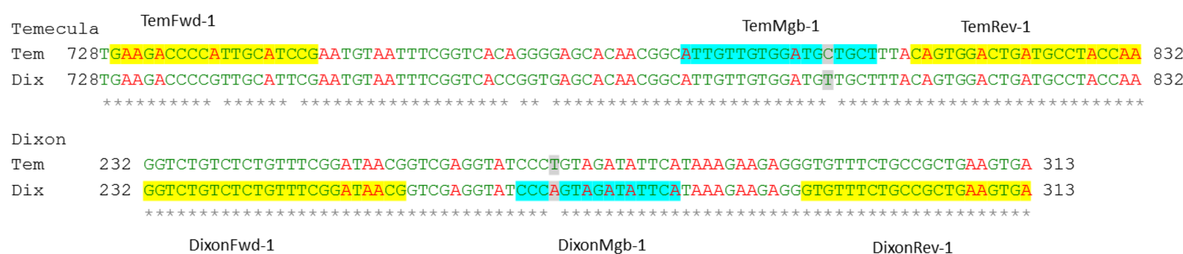


Fig. 1. Gyrase B sequence alignment showing the region of amplification and primers/probe attachment. Yellow indicates primer binding sites, blue indicates probe binding sites and grey indicates mismatch

II. MATERIALS AND METHODS

A. Bacterial Culture and DNA Extraction

Both the Temecula and Dixon isolates were cultured on either PD3 or PWG medium at 28ºC for 7-10 days. Cells were scraped using a sterilized loop and DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Hilden, Germany).

B. Primers and Probe Design

GyraseB sequences of both Temecula and Dixon isolates were taken directly from the NCBI database. These sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to determine the mismatches between the two sequences. Sequences of probes were selected in such a way that there is mismatch between the probe and the DNA of the non-specific isolate. Basic requirements for primer and probe design such as thermodynamic properties, secondary structure formation, primer-primer and primer-probe interactions were evaluated using the IDT Oligoanalyzer 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/Default.aspx).

C. DNA Quantitation and Serial Dilution

Genomic DNA concentrations (gm/µl) were estimated using a spectrophotometer (Nanodrop ND-1000) in triplicate, and DNA copy numbers per µl were determined using the formula:

$$\left(\frac{X}{\text{Target genome size in basepairs}} \times \frac{1}{660}\right) \times 6.022 \times 10^{23} = Y \text{ copynumber}/\mu l$$

After determining DNA concentrations, eight 10-fold serial dilutions ranging from 3.66×10^{7} to 3.66 copy number/µl for the Temecula isolate and 3.52×10^{7} to 3.52 copy number/µl for the Dixon isolate were prepared. These dilutions were used as standards for rt-PCR.

D. Detection of X. Fastidiosa and Formation of Dilution curves

ITS-specific primers and probes described in Schaad et al. [15] were used to run rt-PCR to detect and confirm *X. fastidiosa* and to form standard curves of the dilutions. The 20µl reaction was performed in 0.1 µl strip tubes containing 10 µl 2X IQ Supermix (Biorad) (100mM KCl, 40 mM Tris HCl, 1.6 mM dNTPs, iTaq DNA polymerase 50 units/ml, and 6mM $MgCl_2$), 100nM forward primer, 200 nM reverse primer, and 200nM Taqman probe. The PCR mixture also included 5.8 µl of PCR-grade water and 2 µl of template DNA. The rt-PCR was performed on the Rotor Gene 3000 (Qiagen, Hilden, Germany) at 95ºC for 3 minutes for enzyme activation followed by denaturation at 95ºC for 15 seconds, and extension and annealing at 62ºC for 1 minute. The PCR was run for 40 cycles.

E. RT-PCR Conditions for MGB Probes

The rt-PCR reaction, using the MGB probes, was also performed on the Rotor Gene 3000. The 20 µl reaction mixture contained 2 µl of template DNA and 5.4 µl of PCR grade water with the IQ supermix as mentioned above. The 500 nM forward and reverse primers for the Temecula and Dixon isolates were used along with 80nM of MGB-probe. The rt-PCR for each isolate was run separately.

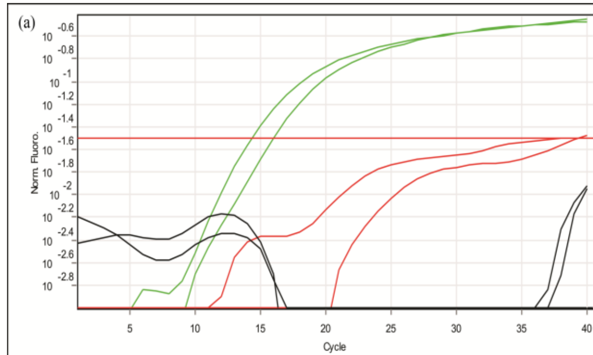
The rt-PCR conditions used for the Dixon isolate were 95ºC for 10 minutes followed by 40 cycles of 95ºC for 15 seconds, and 61ºC for 60 seconds. The Temecula isolate-specific run was performed at 95ºC for 10 minutes followed by 15 seconds at 95ºC then 60 seconds at 68ºC for 40 cycles.

The Dixon isolate-specific rt-PCR included all the serial dilutions of the Dixon DNA as positive control, the highest concentration of the Temecula DNA as a negative control, as well as a non-template control. The Temecula-specific run had all Temecula DNA concentrations as a positive control, the highest

concentration of Dixon DNA as negative control, as well as a non-template control.

F. Detection of a Strain From the Mixture Using the MGB probes

Different concentrations of the Dixon and the Temecula isolates were mixed together as shown in Tables I and II. Twenty µl of the reaction mixture were run on the Rotor Gene 3000 using 2 µl of mixed DNA as a template. Two separate runs for each Temecula or Dixon isolate were performed. The Temecula and the Dixon-specific primer-MGB probes were used separately for each run and PCR conditions were kept the same as mentioned above.



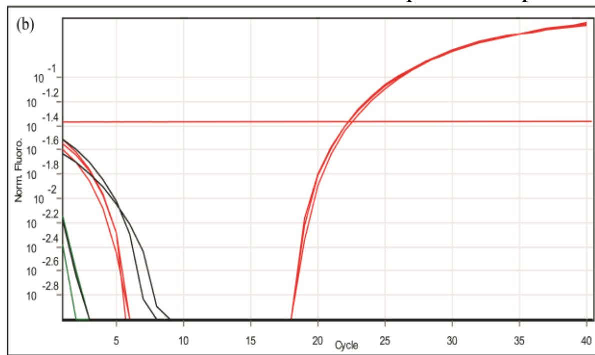
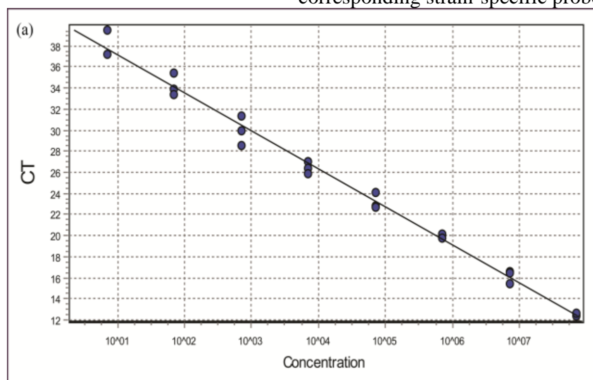


Fig. 2. Graph showing detection of (A) Dixon isolate DNA ( ) (B) Temecula isolate DNA ( ) using gyrase B based primers with corresponding strain-specific probe; Non-template control (NTC) ( )



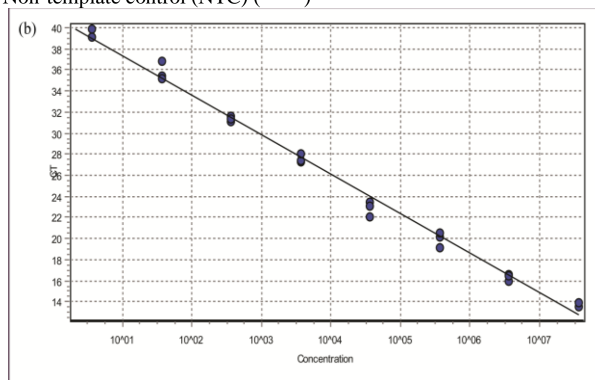


Fig. 3. Standard curve obtained from serially diluted (A) Dixon isolate and (B) Temecula isolate DNA using gyrase B based primers and MGB probes

III. RESULTS

A. Specificity of RT-PCR

The MGB probes were able to differentiate DNA from the Dixon and Temecula isolates (Figs 2A and B). The specificity of the MGB probes was confirmed by electrophoretic analysis of rt-PCR products. DNA of desired length was observed with both the Temecula and the Dixon templates, with the isolate-specific primers-probes (results not shown). These results confirmed that the primers did not discriminate between two DNA's and specificity observed in the rt-PCR was due to the action of the probes and not the primers.

The specificity of these probes was also tested *in silico* with other isolates of PD and ALS strains and we found that the Temecula MGB probe can bind to and detect DNA of all other isolates of the PD strain while the Dixon MGB probe can bind to and detect DNA of all ALS isolates.

B. Sensitivity and Quantification Limit of the Assay

The detection limit of the assay was determined by running rt-PCR of eight 10-fold serial dilutions of genomic DNA ranging from 3.66×10^{7} to 3.66 copy number/µl for the Temecula isolate and 3.52×10^{7} to 3.52 copy number/µl for the Dixon isolate. It took 38.32 cycles to detect 3.52 copy number/µl of the Dixon isolate and 39.51 cycles to detect the Temecula isolate (Figs 3A and B).

The assay sensitivity to detect 3 copy numbers per µl (10^{-5} ng/µl) is comparable with the rt-PCR developed by Schaad et al. (2002), with the added benefit of discriminating between different *X. fastidiosa* Strains (Figs 4A and B).

C. Probe Specificity

The ability of MGB-probes to detect DNA of a given isolate from a mixture of two isolates was

investigated. The DNA of both the Temecula and Dixon isolates was serially diluted and mixed together as shown in Tables I and II. The rt-PCR was then performed to detect one strain from the mixture. The probes demonstrated an ability to discriminate DNA of both isolates from the mixture as well as to detect each isolate correctly. The Temecula specific MGB probe demonstrated very high sensitivity of detection with the DNA concentrations of 5.05×10^{-5} ng/µl (=18.3copy number/µl) detectable when mixed with 50.5 ng/µl (1.76×10^{7}copy number/µl) of the Dixon isolate DNA, thereby giving 10^{6}- fold resolution (Fig. 5A, Table I). So far, based on review of published literature, this is the first reported instance where rt-PCR has been used to detect DNA of one strain from a mixture of two closely related strains.

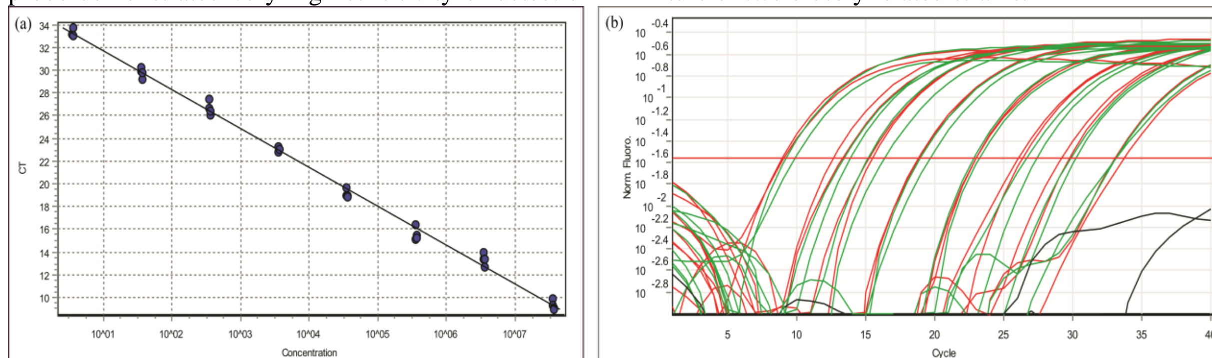


Fig. 4. Graph showing (A) standard curve and (B) detection of both the Temecula ( ) and the Dixon ( ) isolate DNA using ITS based primers and probes, NTC ( ).

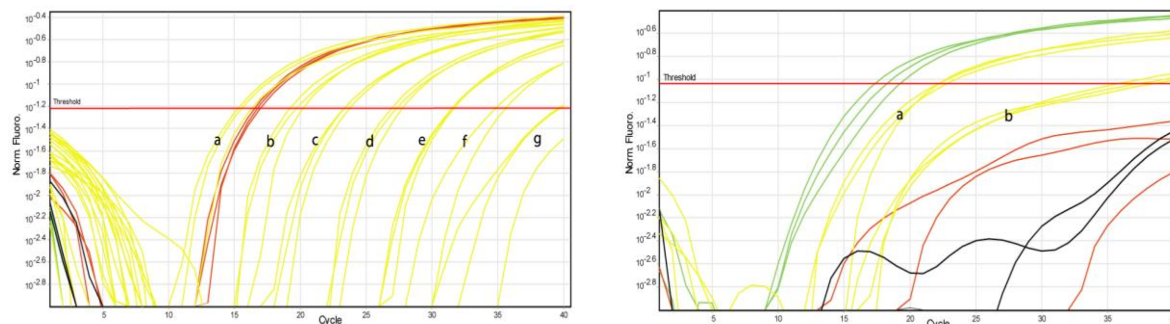


Fig. 5(A). Detection of serially diluted Temecula isolate DNA from a mixture using strain-specific MGB probes: ( ) Temecula isolate, ( ) Dixon isolate, ( ) mixture of DNA, ( ) NTC; Serial dilutions used (cells/µl): a=3.66×10^{7}, b=3.66×10^{6}, c=3.66×10^{5}, d=3.66×10^{4}, e=3.66×10^{3}, f=3.66×10^{2}, g=36.6; Concentration of Dixon DNA used 3.52×10^{7}cells/µl. (B) Detection of serially diluted Dixon isolate DNA from a mixture using strain-specific MGB probes: ( ) Dixon isolate, ( ) Temecula isolate, ( ) mixture of DNA, ( ) NTC; Serial dilutions used (cells/µl): a=3.52×10^{6}, b=3.52×10^{5}; Concentration of Temecula DNA used 3.66×10^{6}cells/µl.

The Dixon specific MGB probe gave only a 10-fold resolution when DNA of two isolates was mixed together (Fig. 5B, Table II). Various primers, probe concentrations and temperature regimens were tried without successfully increasing the resolution of the Dixon-specific run.

IV. Discussion

Disease development in *X. fastidiosa*- infected plants is determined by both pathogen strain and the number of CFU's of the pathogen. It has been reported that petioles from symptomatic plants have 49-fold increase in clogged xylem vessels than those of asymptomatic plants [20]. The development of strain-dependent disease in different plants has been reported by Almeida and Purcell [16], who showed that the ALS strain of *X. fastidiosa* colonized grape plants but could not multiply in enough number to cause PD while the PD strain multiplied in enough number in almond plants to cause ALS. GWSS also showed variations in its association with different *X. fastidiosa* strains. Almeida and Purcell [17] reported that the GWSS transmitted the PD strain three times more efficiently than the ALS strain. Also different strains have been found to co-exist in the same orchard [21]. Hence, quantification as well as accurate detection of *X. fastidiosa* strains in different plants is important to understand disease epidemiology and to anticipate extent and significance of disease development in an area.

In the present study we developed an rt-PCR system to distinguish between the Temecula and Dixon isolates of *X. fastidiosa*. We used dual-labeled MGB probes that gave higher specificity and accuracy than other available PCR based methods [18], [19] and this higher sensitivity enables these probes to discriminate between Temecula and Dixon isolates based on a single base-pair mismatch. In addition to the high specificity achieved by the MGB-probes, the rt-PCR was sensitive enough to detect 3.52 copy number/µl (1.01×10^{-5} ng/µl), which is in the same range achieved by Schaad et al. [15].

We also detected the DNA of one isolate when mixed with the DNA of another isolate. To our knowledge this is the first report of the use of rt-PCR to detect and quantify DNA of one isolate from a mixture of DNA. When Temecula-specific rt-PCR was employed to detect the Temecula isolate (Table I), 5.05×10^{-5} ng/µl (=18 copy number) of Temecula isolate DNA was detectable from a mixture containing 50.5 ng/µl (=1.7×10^{7} copy number) of Dixon isolate DNA (Table I). However, the Dixon specific rt-PCR gave 10-fold resolution (Table II). Initially we thought that the higher sensitivity of the Temecula specific rt-PCR might be due to mismatches present near the 3'end of the forward primer of the Temecula isolate and the gyraseB sequence of the Dixon isolate (Fig. 1) resulting in no PCR product. However, a DNA band of desired length was observed when the Dixon DNA was used as template DNA with Temecula specific primers (results not

TABLE I. DETECTION OF TEMECULA ISOLATE DNA WHEN MIXED WITH DIXON ISOLATE DNA USING TEMECULA-SPECIFIC MGB-PROBE

Temecula (cells/µl)	Dixon (cells/µl)							
	3.52	3.52×10^{1}	3.52×10^{2}	3.52×10^{3}	3.52×10^{4}	3.52×10^{5}	3.52×10^{6}	3.52×10^{7}
3.66	+	+	+	+	+	+	+	-
3.66×10^{1}	+	+	+	+	+	+	+	+
3.66×10^{2}	+	+	+	+	+	+	+	+
3.66×10^{3}	+	+	+	+	+	+	+	+
3.66×10^{4}	+	+	+	+	+	+	+	+
3.66×10^{5}	+	+	+	+	+	+	+	+
3.66×10^{6}	+	+	+	+	+	+	+	+
3.66×10^{7}	+	+	+	+	+	+	+	+

"+"= detectable
"-"= non-detectable

TABLE II. DETECTION OF DIXON ISOLATE DNA WHEN MIXED WITH TEMECULA ISOLATE DNA USING DIXON-SPECIFIC MGB-PROBE

Temecula (cells/µl)	Dixon (cells/µl)							
	3.52	3.52×10^{1}	3.52×10^{2}	3.52×10^{3}	3.52×10^{4}	3.52×10^{5}	3.52×10^{6}	3.52×10^{7}
3.66	+	+	+	+	+	+	+	-
3.66×10^{1}	+	+	+	+	+	+	+	+
3.66×10^{2}	-	+	+	+	+	+	+	+
3.66×10^{3}	-	-	+	+	+	+	+	+
3.66×10^{4}	-	-	-	+	+	+	+	+
3.66×10^{5}	-	-	-	-	+	+	+	+
3.66×10^{6}	-	-	-	-	-	+	+	+
3.66×10^{7}	-	-	-	-	-	-	+	+

"+"= detectable
"-"= non-detectable

shown). This confirmed that the sensitivity was due to the Temecula-specific MGB probe and demonstrates that the MGB probes can be used to detect the presence of a single strain DNA from a mixture. MGB, when attached to the 3' end of the probe increases the Tm resulting in an increased specificity.

We performed two separate runs to distinguish between DNA of Temecula and Dixon isolates from the mixture of DNA of both the isolates. We could not run the multiplex rt-PCR because both the Temecula and the Dixon specific MGB probes had only one kind of attached fluorophore (FAM). It would have been better if two different fluorophores were attached to the probes, which would allow for differentiation between two DNAs in single multiplex rt-PCR. Other dyes were incompatible with the rotor-gene machine and we were not able to get positive result with other dyes.

In conclusion, we have developed a highly specific and sensitive tool that can be used for early

and more accurate detection of different strains of *X. fastidiosa,* either individually or from a DNA mixture.

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